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# Highly sensitive and specific gas chromatographic–tandem mass spectrometric method for the determination of trace amounts of antipyrine metabolites in biological material

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**Abstract**

A highly sensitive and specific gas chromatographic–tandem mass spectrometric method was developed for the determination of the antipyrine (INN: phenazone) metabolites, norantipyrine, 4-hydroxyantipyrine and 3-hydroxymethylantipyrine, in biological material. Deuterated analogues of the metabolites were used as internal standards. The method has a limit of quantitation of 5 ng per sample for the determination of norantipyrine, 4-hydroxyantipyrine and 3-hydroxymethylantipyrine with coefficients of variation of 19.4, 14.6 and 20.7%, respectively. Precision and accuracy are good over the whole range measured (5–500 ng/sample) with a coefficient of variation, respectively error of determination $\leq 20\%$. Due to its high sensitivity the method can be used to study the formation of these metabolites in microsomal preparations containing only 100 $\mu$g of protein.

## 1. Introduction

Antipyrine has been widely used to study the influence of age, disease, drugs, hereditary and environmental factors on drug metabolism [1–3]. For this purpose changes in the plasma or saliva clearance have been mainly used. The drug is extensively metabolized and C-hydroxylation at position 4 [4] and 3-methyl [5] and N-dealkylation [6] are the major metabolic pathways (Fig. 1) accounting for approximately 70% of the dose [7]. Approaches using plasma clearance data or clearance-to-metabolite data to predict how subjects will metabolize other drugs have been disappointing since the determination of antipyrine biotransformation was predictive only for few drugs [1–3,8]. This is not surprising in view of the multiplicity of drug metabolizing enzymes, i.e. cytochrome P450 enzymes where 17 CYPs[1] involved in human drug metabolism have been identified. Although these P450s have a wide substrate specificity, some drugs are preferentially metabolized by one enzyme. It could be

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[1] Abbreviations: AP = antipyrine; C.V. = coefficient of variation; CYP = cytochrome P450 enzyme; DHMAP = [$^2H_3$]-3-hydroxymethylantipyrine; DNORA = [$^2H_3$]norantipyrine; DOHAP = [$^2H_4$]-4-hydroxyantipyrine; HMAP = 3-hydroxyantipyrine; INN = international nonproprietary name; MBDSTFA = N-methyl-*tert.*-butyldimethylsilyltrifluoroacetamide; NORA = norantipyrine; OHAP = 4-hydroxyantipyrine.

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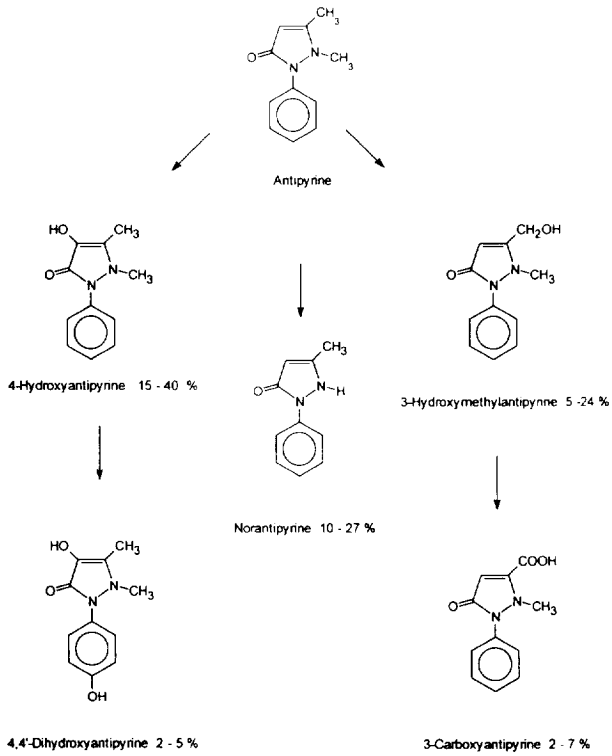


Fig. 1. Phase I metabolites of antipyrine in humans. Numbers indicate the percentage of dose excreted into urine.

that for the formation of each of the antipyrine metabolites a different enzyme might be responsible. So far the cytochrome P450 enzymes involved in antipyrine metabolism have not been identified, a prerequisite for using antipyrine and its metabolites as an appropriate measure of drug metabolism.

Since in in-vitro systems only trace amounts of metabolites are formed, a highly sensitive method for the determination of the metabolites is required. The methods available have a limit of quantitation for the determination of norantipyrine (NORA), 4-hydroxyantipyrine (OHAP) and 3-hydroxymethylantipyrine (HMAP) of 5, 3 and 3 $\mu$g/ml, respectively [7,9–11], which is not sufficient to carry out incubations in human liver microsomes at low substrate concentrations. One radiometric HPLC method is published that had a limit of quantitation for the determination of NORA, OHAP and HMAP of 70, 25 and 25 ng/ml, respectively [12]. Gas chromatographic–mass spectrometric methods published so far have much lower sensitivity with a limit of quantitation being higher than 50 ng of the metabolites per sample [13,14]. Therefore we have developed a highly sensitive and specific coupled gas chromatographic–tandem mass spectrometric method using stable-isotope labelled metabolites as internal standards for the determination of antipyrine metabolites in biological material, i.e. in human liver microsomes.

## 2. Experimental

### 2.1. Materials

Norantipyrine and 4-hydroxyantipyrine were purchased from Sigma (Deisenhofen, Germany) and antipyrine (Ph. Eur.) (INN: phenazone) was obtained from Fluka (Neu-Ulm, Germany). 3-Hydroxymethylantipyrine was synthesized as described by Nakagawa et al. [14], Buijs et al. [15] and Zietz et al. [16]. [$^2H_3$]-3-Hydroxymethylantipyrine (DHMAP) was synthesized via [$^2H_3$]Phenazone starting with norantipyrine according to the method described [17]. [$^2H_3$]Norantipyrine (DNORA) and [$^2H_4$]-4-hydroxyantipyrine (DOHAP) were obtained by heating the respective metabolite with deutero sulfuric acid under reflux in an argon atmosphere. [$^2H_3$]Norantipyrine was purified by sublimation. [$^2H_4$]-4-Hydroxyantipyrine was crystallized from diethyl ether. The structure and purity of the products were verified by elementary analysis and $^1$H NMR spectroscopy.

All solvents used were of HPLC quality, chemicals of analytical grade: methanol (Baker, Groß-Gerau, Germany), NADPH (Sigma), Silicar CC-4 Special (Mallinckrodt, Paris, KY, USA), N-methyl-*tert.*-butyldimethylsilyltrifluoroacetamide (MBDSTFA) (Macherey-Nagel, Düren, Germany), acetonitrile (Rathburn, Zinsser Analytic, Frankfurt-am-Main, Germany). All other chemicals and solvents were of analytical grade and purchased from Merck (Darmstadt, Germany). Only silanized glassware was used.

Dichloromethane (Promochem, Wesel, Germany) was purified with basic alumina (Alumina B-Super I, ICN Biomedicals, Eschwege, Germany). Ethyl acetate (Merck) was distilled before use. Silicar columns were prepared by filling 4-ml reservoirs W/frits (Varian, Harbor City, CA, USA) with 2 g of Silicar and washing the column with 10 ml of methanol and 10 ml of ethyl acetate.

Human liver microsomes and rat liver microsomes were prepared as described by Meier et al. [18]. Protein concentration was determined according to Lowry et al. [19].

### 2.2. Reference and stock solutions

The deuterated analogues were used as internal standards of the respective metabolites. Stock solutions of the metabolites and the internal standards (1–5 mg/ml) were prepared in methanol and stored at −80°C. Dilutions containing 5 ng/μl of the metabolites and their internal standards were prepared in aqueous $Na_2S_2O_5$ solution (1.5 mg/ml for the references and 20.25 mg/ml for the internal standards) to prevent the substances from oxidation. These solutions were then divided into aliquots and stored at −20°C until use.

### 2.3. Incubation, extraction and derivatization

Incubations were carried out in Eppendorf Caps for 15 min at 37°C. The incubation solution consisted of 250 μl potassium phosphate buffer (0.1 mol $l^{-1}$, pH 7.4), $MgCl_2$ 6 mmol $l^{-1}$, NADPH 5 mmol $l^{-1}$, antipyrine 0.1 to 100 mmol $l^{-1}$ and microsomal protein 200 μg. The reaction was started by addition of NADPH and stopped by adding 300 μl of 2-propanol and cooling on ice. A 100-ng quantity of each internal standard dissolved in 20 μl was added to the sample and the samples mixed on a vortex-mixer. Subsequently the samples were pipetted into 4-ml glass vials containing 2.7 ml of dichloromethane. The vials were mixed for 10 min in an overhead mixer, centrifugated at 650 *g* for 10 min and the aqueous phase was discarded.

For the determination of 4-hydroxyantipyrine and 3-hydroxymethylantipyrine 1 ml of the organic phase was evaporated to dryness at 30°C under a stream of nitrogen. The residue was derivatized with 50 μl of MBDSTFA–acetonitrile (1:9, v/v) and an aliquot of 2 μl was injected into the GC–MS–MS. For the determination of norantipyrine the remainder of the organic phase was evaporated. The residue was dissolved in 100 μl of ethyl acetate and applied to a Silicar column which had been conditioned by rinsing with 10 ml of methanol and subsequently 10 ml of ethyl acetate. The column was eluted with 6 ml of methanol–ethyl acetate (1:9, v/v). The first 2 ml of the eluate were discarded, the next 4 ml were evaporated and the residue was derivatized with 50 μl of MBDSTFA–acetonitrile (1:9, v/v). An aliquot of 2 μl was injected to the GC–MS–MS.

### 2.4. Calibration curves and quality control samples

Calibration curves and quality control samples were prepared by adding known amounts of the metabolites to boiled rat liver microsomes containing buffer, $MgCl_2$, and NADPH as described above. To these samples 0.1 mmol/l antipyrine was added. Calibration curves were prepared with at least 5 different concentrations over a concentration range of 5–500 ng per sample. The samples were not incubated to avoid non-enzymatic formation of 4-hydroxyantipyrine in aqueous solutions containing iron ions from the rat liver microsomes. Standard curves were obtained by linear regression analysis using peak-area ratios of the respective daughter ions ($y$) and the amounts of the reference substance ($x$). Accuracy and precision were determined in quality control samples. The samples were treated as described above.

### 2.5. Instrumentation and chromatographic conditions

GC–MS–MS analysis was performed on a Hewlett-Packard 5890 II gas chromatograph coupled to a Finnigan MAT TSQ 700 tandem mass spectrometer. The gas chromatograph was

equipped with an autosampler CTC-A200S. Gas chromatographic conditions were: carrier gas helium with a column head pressure of 100 kPa; capillary column: DB 5, 30 m × 0.25 mm I.D., film thickness 0.25 μm (J&W Scientific); splitless injection at 280°C. The initial temperature of 80°C was held for 1 min and then increased at 30°/min to 300°C and kept at this temperature. Under these conditions the retention times for NORA, OHAP and HMAP were 7.3, 8.5 and 9.3 min, respectively. Mass spectrometry was performed in the electron-impact daughter-ion mode. MS conditions were: source temperature 150°C, electron energy 70 eV, emission current 200 μA, argon collision cell pressure 160 mPa. The molecular ions of the respective derivatives were used as parent ions. The following masses were used (*m/z* parent ion:*m/z* daughter ion): NORA (288:191); DNORA (291:194); OHAP (318:246); DOHAP (322:250); HMAP (318:233); DHMAP (321:236).

The parameters of Michaelis–Menten kinetics were calculated from the rates of formation at different substrate concentrations and the substrate concentrations using the nonlinear regression software programme Enzfitter (Biosoft, Cambridge, UK).

## 3. Results and discussion

The method developed has a high sensitivity and is able to detect 1 ng of NORA, OHAP and HMAP in human liver microsomes at a signal-to-noise ratio >3. Calibration curves have excellent linearity over the entire range measured (5–500 ng; ≈ 10–2500 pmol) with *r*-values better than 0.998 (Table 1). During a period of nine months variability of the calibration curves was very small, the slopes remaining almost constant (coefficient of variation, C.V. <10%), indicating a good reproducibility. The intercepts varied depending on the content of antipyrine in the samples. The relative error of the standards was <16% at 5 ng, decreasing at higher concentrations. The limit of quantitation was 5 ng (29 pmol, resp. 25 pmol) of each metabolite per sample. In nine samples containing 5 ng of NORA, OHAP and HMAP the mean amounts found were 4.3 ± 0.8, 4.8 ± 0.7 and 4.3 ± 0.9 ng, respectively with C.V.s of 19.4, 14.6 and 20.7%. Accuracy, within-day and between-day precision were excellent (Table 2). The relative error of between-day samples was better than 5% for samples containing 10 or 500 ng of each metabolite. At a concentration of 100 ng the relative error was below 2%.

The excess of antipyrine in the incubation media interfered with the determination of norantipyrine since a small part of antipyrine reacted with MBDSTFA yielding derivatized norantipyrine. Therefore antipyrine had to be removed prior to determination of norantipyrine. The samples were divided and norantipyrine could be separated by chromatography on Silicar columns from antipyrine prior to derivatization as described above. Antipyrine as well as 4-hydroxyantipyrine and 3-hydroxymethylantipyrine were retained whereas norantipyrine was eluted. 4-Hydroxyantipyrine and 3-hydroxymethylantipyrine were measured besides antipyrine in the other part of the samples.

Table 1
Calibration curves for antipyrine metabolites

| Metabolite | Slope (Mean ± S.D.) | Intercept (Mean ± S.D.) | Correlation coefficient (range) |
|---|---|---|---|
| NORA | 0.01998 ± 0.00194 | 0.15065 ± 0.10568 | 0.9994–1.000 |
| OHAP | 0.02560 ± 0.00219 | 0.18208 ± 0.22169 | 0.9994–1.000 |
| HMAP | 0.01082 ± 0.00094 | 0.02732 ± 0.01865 | 0.9982–1.000 |

*n* = 28.

Table 2
Assay variability for the determination of antipyrine metabolites in liver microsomes

| Compound | Found | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 10 ng ($n = 5$) | | | 100 ng ($n = 5$) | | | 500 ng ($n = 5$) | | |
| | Mean (ng) | S.D. | C.V. (%) | Mean (ng) | S.D. | C.V. (%) | Mean (ng) | S.D. | C.V. (%) |
| *Within-day precision* | | | | | | | | | |
| NORA | 10.4 | 0.5 | 4.7 | 103.1 | 4.1 | 4.1 | 491.8 | 9.1 | 1.8 |
| OHAP | 11.9 | 0.7 | 7.0 | 99.7 | 5.8 | 5.8 | 444.5 | 26.0 | 5.2 |
| HMAP | 10.2 | 1.3 | 12.7 | 114.9 | 4.2 | 4.2 | 535.2 | 16.4 | 3.3 |
| | Found | | | | | | | | |
| | 10 ng ($n = 29$) | | | 100 ng ($n = 42$) | | | 500 ng ($n = 22$) | | |
| | Mean (ng) | S.D. | C.V. (%) | Mean (ng) | S.D. | C.V. (%) | Mean (ng) | S.D. | C.V. (%) |
| *Between-day precision* | | | | | | | | | |
| NORA | 9.6 | 2.5 | 20.5 | 100.3 | 8.8 | 6.1 | 480.0 | 36.5 | 5.4 |
| OHAP | 10.4 | 2.3 | 19.6 | 102.0 | 10.5 | 7.9 | 489.6 | 38.8 | 5.3 |
| HMAP | 10.5 | 1.4 | 10.5 | 100.0 | 7.0 | 5.4 | 479.0 | 35.8 | 5.3 |

S.D. = standard deviation; C.V. = coefficient of variation.

Due to its high sensitivity the method described is well suited for the determination of the trace amounts of metabolites formed in the microsomal fraction of human liver in vitro. Michaelis–Menten kinetics for the formation of the three metabolites could be calculated from the amounts of metabolites formed during an incubation. An example of the formation of OHAP in human liver microsomes is presented in Fig. 2. $K_m$ values for the formation of NORA, OHAP and HMAP were 14.4, 23.1 and 20.8 mmol $l^{-1}$, respectively. The $V_{max}$ values in this liver were 0.83, 1.40 and 0.59 nmol $mg^{-1}$ $min^{-1}$.

The results obtained in this experiment are in good accordance with the data of Kahn et al. [12] who investigated the Michaelis–Menten kinetics of antipyrine metabolite formation in vitro in liver microsomes from three patients using a radiometric HPLC method. The $K_m$ values found by Kahn et al. ($K_m$: NORA: 5.9 ± 0.6 mmol $l^{-1}$; OHAP: 7.3 ± 1.1 mmol $l^{-1}$; HMAP: 9.0 ± 1.3 mmol $l^{-1}$) are of the same order of magnitude.

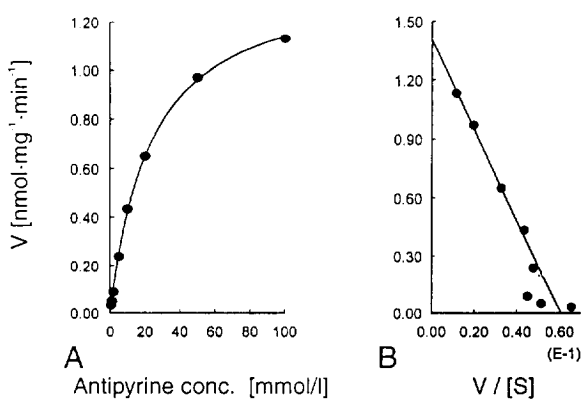


Fig. 2. (A) Michaelis–Menten kinetics of 4-hydroxyantipyrine formation in human liver microsomes. (B) Eadie–Hofstee plot; 200 μg microsomal protein, 15-min incubation time. $V_{max}$: 1.40 nmol $mg^{-1}$ $min^{-1}$; $K_m$: 23.1 mmol $l^{-1}$.

The use of the assay developed allows the characterization of antipyrine biotransformation in human liver microsomes. This enables the in-vitro identification of the cytochrome P450 enzymes involved in the metabolism of the model drug antipyrine. Studies identifying the cytochrome P450 enzymes involved in antipyrine metabolism are currently underway.

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